XL. AN IMPROVED METHOD FOR THE COLORIMETRIC DETERMINATION OF PHOSPHATE

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ALL the known colorimetric methods for the determination of phosphate are liable to errors arising from (*a*) slight changes in acidity and concentration of reagents, and (*b*) the presence of interfering substances (e.g. fluorides, nitrites etc.). The following method is almost entirely independent of these disturbing factors, and possesses the added advantage of being more sensitive than those previously described.

EXPERIMENTAL

Principle of the method

The method is based on the ready solubility of the reducible phosphomolybdic acid in *iso*butyl alcohol. It consists essentially of the reduction of phosphomolybdic acid to the blue complex by shaking the alcoholic extract with an acidified aqueous solution of stannous chloride. Since the extraction occurs readily over a wide acid range (0·05–1·5 *N* H_2SO_4), and is not affected by the presence of excess of molybdic acid, considerable variation in the concentrations of these reagents is permissible without any danger of interference with the ultimate colour production. The concentration of reducing agent may also vary over a wide range, while the effects of interfering substances are prevented by the use of high concentrations of molybdate and reducing agent.

Solutions required

(1) 10 *N* sulphuric acid (approx.): 28 % conc. H_2SO_4.

(2) *N* sulphuric acid (approx.): sol. (1) diluted 10 times.

(3) 5 % ammonium molybdate (Kahlbaum "for analysis"). This solution must be kept in a bottle, coated inside with paraffin wax, to prevent the slow formation of a silicon compound of molybdic acid which yields a blue colour on reduction.

(4) Stock solution of stannous chloride: 10 g. stannous chloride is dissolved in 25 ml. conc. HCl (kept in a brown glass-stoppered bottle).

(5) Dilute stannous chloride solution: sol. (4) is diluted 200 times with 1 *N* H_2SO_4 (sol. (2)). It must be made up fresh when required.

(6) *iso*Butyl alcohol.

(7) Ethyl alcohol.

The following phosphate solutions are required for comparison:

A. Stock solution: 2·193 g. KH_2PO_4 in 500 ml. water (=1 mg. P per 1 ml.).

B. Solution containing 10γ per ml.: sol. A diluted 100 times.

C. Solution containing 1γ per ml.: sol. A diluted 1000 times.

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Determination of phosphate in 10 ml. of total volume of fluid

A. *For inorganic phosphorus.* If the solution to be tested is strongly acid or alkaline, it must first be neutralized. Then 5 ml. of this are measured into a separating funnel and the following added: 0·5 ml. of 10*N* H_2SO_4, 2 ml. of dist. water, 2·5 ml. of 5% ammonium molybdate and 10 ml. of *iso*butyl alcohol. The mixture is shaken for 1 or 2 min. and the aqueous layer is discarded. The alcoholic solution is washed by shaking with two lots of 5 ml. of *N* H_2SO_4, and then shaken with about 15 ml. of the dilute stannous chloride solution for about 30 sec. and the aqueous layer discarded. The blue solution is poured into a 10 ml. measuring flask; the separating funnel is washed with ethyl alcohol and the solution made up to the mark with the washings. (The ethyl alcohol is necessary to bring into solution emulsified droplets of water.)

B. *For inorganic phosphorus in biological fluids.* The proteins are first removed by the addition of an equal volume of 12% trichloroacetic acid and centrifuging. Of the supernatant fluid, 5 ml. are measured into a separating funnel, and the procedure then continued as described above, except that 0·3–0·4 ml. of 10*N* H_2SO_4 is added instead of 0·5 ml.

C. *For organic phosphorus.* When the phosphorus is in organic combination, the incineration method of King [1932] is recommended, 1 ml. of 60% perchloric acid being used for the incineration (with addition of one or two drops of H_2O_2 if very much organic material is present). The colourless solution thus obtained is made up to 5 ml. and tested as described above, except that no 10*N* H_2SO_4 is added.

The intensities of blue colour may either be determined by an ordinary colorimeter, or by means of a "Spekker" Photoelectric Absorptiometer, after calibrating the instrument against standard amounts of phosphate.

The safe ranges of concentration of reagents and the effects of interfering substances were studied, and the results compared with those obtained by the methods of Fiske & Subbarow [1925] and Kuttner & Cohen [1927]. The results, which are summarized in Tables I and II, illustrate the wide margin of safety of the present method.

Micro-determination of phosphate

The above method can be made 10 times more sensitive by reducing all the quantities to a total volume of 1 ml. The only requirements are some micro-pipettes, a special small mixing vessel in the place of the separating funnel, and a suitable colorimeter for examining small amounts of fluids.

A micro-mixing vessel suitable for the purpose is shown in Fig. 1. It consists of a bulb of about 10 ml. capacity with two lengths of very fine capillary tubing attached. One of these is bent and the end ground to a fine point, through which fluid is sucked into the vessel, while the other is connected to a mouth-piece by rubber tubing.

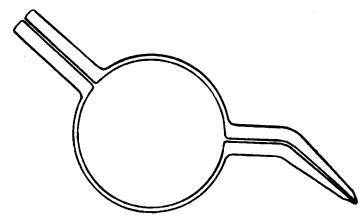

Fig. 1.

The following substances are measured into a small crucible by means of micro-pipettes: 0·05 ml. of 10 *N* H_2SO_4, 0·25 ml. of 5% ammonium molybdate and 0·5 ml. of the solution to be tested. The mixture is sucked up into the mixing vessel, the crucible is rinsed with 0·2 ml. of dist. water and this is also taken up into the vessel, after which 1 ml. of *iso*butyl alcohol is sucked up and the vessel shaken, the ends being held closed with two fingers. The subsequent procedure is similar to that

in the macro-method, except that about 1 ml. of N H_2SO_4 and 1·5 ml. of dilute reducing agent respectively are used, and the final volume made up with ethyl alcohol in a 1 ml. measuring flask.

For the present work, the colour intensities were estimated by means of a "Spekker" Photoelectric Absorptiometer specially adapted and fitted with vessels of 1 ml. capacity. The results obtained by the micro-method compared very favourably with those obtained by the standard method.

Discussion

In attempting to assess the usefulness of the present method, comparisons were made with the two most frequently used methods, namely that of Fiske & Subbarow and that of Kuttner & Cohen.

As regards sensitivity, the depth of colour developed by the present method for a given amount of phosphate was found to be at least 4 times that of Fiske & Subbarow, and of the same order as that of Kuttner & Cohen. With the latter method, however, the control is usually slightly coloured so that the determination of the faint colours produced by less than 5γ of phosphorus per 10 ml. is not reliable. With the present method, no colour develops in the control, so that as little as 1γ of phosphorus can be determined with accuracy. The method is reliable for quantities up to 100γ of phosphorus per 10 ml.

In the previous communication [Berenblum & Chain, 1938] substances interfering with the colorimetric determination of phosphate were classified as follows:

A. Substances which alter the acidity of the medium, i.e. acids, alkalis and buffers.

B. Substances which combine readily with molybdic acid to form complexes which are difficult to reduce, e.g. fluorides, tartrates, citrates etc.

C. Substances which interfere with the reduction of phosphomolybdic acid, e.g. nitrites, hypochlorites etc.

Table I. *Safe limits of variation in amounts of reagents*

Reagent	Method of Fiske & Subbarow	Method of Kuttner & Cohen	Present method*
Acid (H_2SO_4)	±30%	±10%	+200% − 90
Molybdate	±30	±10	+100 − 90
Reducing agent	±30	±30	+100 − 90

* +100% represents double the concentration, and −90% 1/10 the concentration normally used for the method.

Both the method of Fiske & Subbarow and that of Kuttner & Cohen are very sensitive to interfering substances of groups A and B, the latter being somewhat more susceptible than the former (Tables I and II). Such interferences are almost completely eliminated in the present method by using excess of molybdate at a relatively low acidity (0·5 N). This is made possible by the fact that the excess of molybdate is removed in the aqueous layer prior to reduction.

The method of Kuttner & Cohen is extremely susceptible to interfering substances of group C (Table II) owing to the small amount of reducing agent permitted in the test. The method of Fiske & Subbarow is less sensitive to these

Table II. *Inhibition of colour production*

Inhibitors*	Method of Fiske & Subbarow	Method of Kuttner & Cohen	Present method
Sodium nitrite:			
0·1 *M*	Complete inhib.	Complete inhib.	Slight inhib.
0·01 *M*	Slight inhib.	Complete inhib.	No inhib.
0·001 *M*	No inhib.	Marked inhib.	No inhib.
0·0002 *M*	No inhib.	Slight inhib.	No inhib.
Sodium citrate:			
0·04 *M*	Complete inhib.	Complete inhib.	Moderate inhib.
0·01 *M*	Almost complete inhib.	Marked inhib.	No inhib.
0·004 *M*	Slight inhib.	No inhib.	No inhib.
Sodium oxalate:			
0·04 *M*	Complete inhib.	Complete inhib.	No inhib.
0·01 *M*	Moderate inhib.	Moderate inhib.	No inhib.
0·002 *M*	Slight inhib.	No inhib.	No inhib.
Sodium fluoride:			
0·1 *M*	Complete inhib.	Complete inhib.	No inhib.
0·05 *M*	Moderate inhib.	Moderate inhib.	No inhib.

* The figures in this column represent molar concentrations in the final mixtures.

inhibitors because of the excess of sulphite present in the reducing mixture. In the present method, the effects of these inhibitors are almost completely eliminated by using great excess of reducing agent.

Finally, the discarding of the aqueous layer and the washing of the alcoholic layer with dilute acid prior to reduction, not only helps to eliminate the potential inhibitors, but also prevents the "non-specific" development of blue colour in the control.

Summary

1. An improved method is described for the colorimetric determination of phosphate. It consists of the removal of the reducible phosphomolybdic acid from the aqueous solution by extraction with *iso*butyl alcohol, and its reduction with stannous chloride.

2. The amount of phosphorus which can be accurately determined is 1–100γ by the standard method and 0·1–10γ by the specially designed micro-method.

3. The method is almost completely independent of interfering substances.

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